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Calcium-dependent phospholipid scrambling by TMEM16F

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In all animal cells, phospholipids are asymmetrically distributed between the outer and inner leaflets of the plasma membrane¹. This asymmetrical phospholipid distribution is disrupted in various biological systems. For example, when blood platelets are activated, they expose phosphatidylserine (PS) to trigger the clotting system^{2,3}. The PS exposure is believed to be mediated by Ca²⁺-dependent phospholipid scramblases that bidirectionally transport phospholipids^{1,4}, but its molecular mechanism has remained elusive. Here we found that when a mouse B-cell line, Ba/F3, was treated with a Ca²⁺-ionophore under Ca²⁺-low conditions, it reversibly exposed PS. Using this property, we established a Ba/F3 subline that strongly exposed PS by repetitive FACS sorting. A cDNA library was constructed from the subline, and a cDNA that caused Ba/F3 to spontaneously expose PS was identified by expression cloning. The cDNA encoded a constitutively active mutant

of TMEM16F (Transmembrane protein 16F), a protein with eight transmembrane segments⁵. The wild-type TMEM16F was localized on the plasma membrane, and conferred the Ca^{2+} -dependent scrambling of phospholipids. A patient with Scott Syndrome^{6,7}, which results from a defect in phospholipid scrambling activity^{8,9}, was found to carry a mutation at a splice-acceptor site of the TMEM16F gene, causing the premature termination of the protein. We conclude that TMEM16F is an essential component for the Ca^{2+} -dependent PS exposure on the cell surface.

When mouse Ba/F3 cells were treated with 1.0 μM A23187 for 15 min in the presence of 0.5 mM CaCl_2 , the cells underwent necrosis or became propidium iodide (PI)-positive. However, when the same treatment was performed in Ca^{2+} -free condition, most of the cells exposed PS, and the PI-positive population was low (Fig. 1a). Chelating intracellular Ca^{2+} with BAPTA-AM blocked the PS exposure (Fig. 1b), indicating that the process required mobilization of intracellular calcium. This PS-exposure was reversible. That is, treating the PS-exposing cells with BAPTA-AM at 37°C for 5 min (Fig. 1c) or culturing them in Ca^{2+} -free medium at 37°C for 12 h (data not shown), eliminated the PS from the cell surface. These results suggested that under Ca^{2+} -low conditions, A23187 mobilized the intracellular Ca^{2+} , which activated a phospholipid scramblase to expose PS. Upon reduction of the intracellular Ca^{2+} concentration, the phospholipid scramblase lost activity, and flippases returned the PS to the inner leaflet.

To characterize the PS-exposure process, we used its reversible nature under Ca^{2+} -low conditions to establish a cell line that over-exposed PS. Ba/F3 cells were treated with 1.0 μM A23187 in the absence of calcium, and subjected to FACS sorting based on PS exposure. A population (0.5-5%) that showed intense Annexin V staining was collected, cultured for 15 h in Ca^{2+} -free medium, returned to normal medium, and subjected to the next sorting. After this cycle of sorting and expansion was repeated 12

times, the cells (Ba/F3-PS12) exhibited about a 100-fold higher staining with Annexin V than the original Ba/F3 cells (Ba/F3-PS0) upon treatment with 125 nM of A23187 (Fig. 1d). The sorting and expansion was repeated another seven times, and the resulting cell line (Ba/F3-PS19) was used for further studies.

There were two possible causes of the strong PS exposure in Ba/F3-PS19. One was the over-expression or over-activation of phospholipid scramblase, and the other was the inactivation of flippase¹⁰ that transports PS from the outer to inner leaflet of the plasma membranes. To examine which possibility was correct, DsRed-expressing Ba/F3-PS19 was fused with the GFP-labeled parental Ba/F3 (Ba/F3-PS0). The PS-exposure response of the hybrid cells to 1.0 μ M A23187 was similar, or slightly weaker than that of Ba/F3-PS19 (Fig. 1e), suggesting that the phenotype of Ba/F3-PS19 was dominant to that of Ba/F3-PS0, and that the phospholipid scramblase was over-activated in Ba/F3-PS19. To identify the gene responsible for the enhanced phospholipid scramblase activity, a cDNA library (9.3×10^5 clones) was prepared from Ba/F3-PS19, and introduced into the parental Ba/F3. The stably transformed cells were treated with 125 nM A23187, and a population that stained strongly with Annexin V was sorted (Fig. 1f). At the third cycle of sorting and expansion (Library-Derived (LD)-PS3), about 35% of the cells exposed PS without A23187 treatment, and this cell population (LD-PS4) was characterized.

The LD-PS4 cells carried 2-3 different cDNAs, but the TMEM16F cDNA (GenBank NM_175344) was present in two independent experiments, suggesting that TMEM16F caused the PS exposure. The two TMEM16F cDNAs identified in the different experiments contained an A-to-G mutation at nucleotide 1226, which caused an aspartic acid to be replaced by glycine at codon 409 (Fig. 2a). TMEM16A, another member of the TMEM16 family, was recently shown to be a Ca^{2+} -dependent Cl^- channel¹¹⁻¹³. However, the Cl^- channel activity of TMEM16F was low compared to that

of TMEM16A¹⁴. To examine the function of TMEM16F, the wild-type and mutant (D409G) forms of TMEM16F were tagged with Flag or mRFP at C-terminus, and expressed in Ba/F3 or human 293T cells. Western blotting of the cell lysates with anti-Flag showed broad bands at 125 and 500 kDa on SDS-PAGE (Fig. 2b), suggesting that mouse TMEM16F (calculated Mr. 106,000) is glycosylated and/or aggregated. Observation of the 293T cells expressing TMEM16F-mRFP indicated that TMEM16F is at the plasma membrane (Fig. 2c).

Annexin V bound to the Ba/F3 expressing the D409G mutant, but not the wild-type TMEM16F (Fig. 2d), suggesting that the mutant TMEM16F-expressing cells constitutively expose PS. This was confirmed by binding of MFG-E8 that specifically binds to PS^{15,16} (Supplementary Fig. 1). Chelating the intracellular Ca²⁺ by BAPTA-AM reduced the exposed PS level in the mutant TMEM16F-expressing cells (Fig. 2d). When cells expressing the wild-type TMEM16F were treated with A23187, PS was exposed without a lag time, and reached the saturation level more quickly than the vector-transformed Ba/F3 (Fig. 2e). The intracellular Ca²⁺ concentration and the kinetics of the Ca²⁺-influx upon the A23187-treatment was similar among the vector-, wild-type-, and D409G mutant TMEM16F-expressing cells (Supplementary Fig. 2). These results indicated that TMEM16F mediates a Ca²⁺-dependent scramblase activity for PS, and that its D409G mutant is sensitized to respond to the normal intracellular concentration of Ca²⁺ to expose PS.

Phospholipid scramblase mediates bidirectional transfer between plasma membrane leaflets of all phospholipids. The cells expressing the D409G mutant TMEM16F were stained with Ro09-0198 (Supplementary Fig. 3a), a tetracyclic polypeptide that specifically binds phosphatidylethanolamine (PE)¹⁷, indicating that they constitutively exposed PE, a phospholipid that like PS is normally sequestered to the inner leaflet. The treatment of Ba/F3 with A23187 caused the PE-exposure. This

process was accelerated by over-expressing the wild-type TMEM16F (Supplementary Fig. 3b). When NBD-phosphatidylcholine (NBD-PC) was added to the culture, it was quickly internalized by the D409G-mutant-expressing cells (Fig. 2f). That is, among the cell-associated NBD-PC, more than 40% NBD-PC became resistant to the BSA-extraction within 6 min. When the cells expressing the wild-type TMEM16F were treated with A23187, they incorporated NBD-PC faster than the parental cells, and about 40% of the cell-associated NBD-PC was inside of the cells within 4 min (Fig. 2g). Similar results, i.e., constitutive internalization by cells expressing the mutant TMEM16F, and the enhanced A23187-induced incorporation by cells expressing the wild-type TMEM16F, were obtained with NBD-sphingomyelin (NBD-SM)(Supplementary Fig. 4). The internalized NBD-PC and NBD-SM were intact (Supplementary Fig. 5). Dynasore that inhibits dynamin-mediated endocytosis¹⁸ did slightly or not inhibited the internalization of these phospholipids (Supplementary Fig. 6), suggesting that the contribution of endocytosis in the TMEM16F-mediated phospholipid-internalization may not be great.

The expression of the endogenous TMEM16F in Ba/F3 was then knocked down by expressing TMEM16F shRNA. As shown in Fig. 3a and Supplementary Fig. 7, the expression level of TMEM16F mRNA in 5 transformants was reduced to 20-35% of that in the cells expressing the control shRNA. The rate of A23187-induced exposure of PS and PE was reduced in these transformants (Fig. 3b and 3c). Similarly, the uptake of NBD-PC and NBD-SM was slower in the TMEM16F shRNA-transformed cells (Fig. 3d and 3e).

Platelets and other blood cells from patients with Scott syndrome show a defect in their ability to expose PS in response to a Ca^{2+} -ionophore^{7,19}. Kojima et al.²⁰ established B-cell lines from a Scott syndrome patient and the patient's parents. In agreement with previous reports^{8,20}, the patient-derived cells did not expose PS in response to a Ca^{2+} -

ionophore (Fig. 4a). In contrast, A23187 elicited PS exposure in cell lines derived from the patient's parents at the same levels as cell lines from healthy volunteers. An RT-PCR analysis of the TMEM16F mRNA (GenBank NM_001025356) showed that the 5' part (1320 bp) corresponding to exons 1-12 was identical among the patient and the parents, while its 3' half corresponding to exons 11-20 was shorter in the patient than that in the parents (Fig. 4b). A sequence analysis indicated that the cDNA of the patient lacked the 226-bp sequence corresponding to exon 13. Direct sequencing of the chromosomal DNA indicated that the TMEM16F gene of the patient carried a G-to-T homozygous mutation at the splice-acceptor site in intron 12, while both parents were heterozygous for the mutation at this position (Fig. 4c). PCR analysis of the TMEM16F mRNA with primers at exons 12 and 16 showed a 608-bp band from the control and a 382-bp band from the Scott-syndrome patient's cell line (Fig. 4d), indicating that a mutation in the splice acceptor site caused exon 13 to be skipped. This skipping caused a frame shift, which resulted in the premature termination of the protein in exon 14 (Fig. 4e) at the third transmembrane segment of human TMEM16F (Fig. 4f). The nonsense-mediated mRNA decay²¹ may explain the reduced concentration of the exon 13-deleted form of TMEM16F mRNA in the patient's parents (Fig. 4d).

Repeated FACS sorting has been used previously to establish cell lines that over-express a particular cell-surface protein^{22,23}. Here, this method yielded TMEM16F carrying a point mutation that rendered the process extremely sensitive to Ca^{2+} , such that in the cells expressing the mutated TMEM16F, the phospholipid scramblase functioned even in resting cells, in which the cytosolic Ca^{2+} concentration is below 100 nM²⁴. The TMEM16 family, to which TMEM16F belongs, consists of 10 members in human and mouse⁵. The founding member of the family, human TMEM16A, is a Ca^{2+} -dependent Cl^- -channel¹¹⁻¹³. Although the direct binding of Ca^{2+} to TMEM16 members has yet to be demonstrated, the N-terminal region of TMEM16A appears to have a regulatory role²⁵. Similarly, the increased Ca^{2+} -sensitivity of the D409G mutant

suggests that either Ca^{2+} or a Ca^{2+} -sensing molecule binds to this N-terminal region of TMEM16F. The over-expression of TMEM16A in Ba/F3 cells had no effect on the ionophore-induced PS exposure (data not shown), suggesting that different members of this family have distinct functions. The PS exposure or scrambling of phospholipids occurs in other biological processes^{1,4,26-29}, such as the apoptotic cell death, the fusion of muscle, bone or trophoblast cells, and the release of neurotransmitters and microvesicles. It will be interesting to study whether TMEM16F and/or its related members in the TMEM16 family are involved in these processes.

Methods Summary

To reversibly expose PS on the cell surface, Ba/F3 cells were treated at 37°C with A23187 in Ca^{2+} -free condition. The exposed PS was detected by binding of Annexin V at 4°C in the Ca^{2+} -containing Annexin V-binding buffer. A subline (Ba/F3-PS19) of Ba/F3 cells that was extremely sensitive to Ca^{2+} -ionophore-elicited PS exposure was selected by repeating FACS sorting 19 times with FACSAria (BD Bioscience). A cDNA library was established with mRNA from Ba/F3-PS19 in retrovirus vector, and the cDNA (TMEM16F) that renders Ba/F3 to constitutively expose PS was identified by expression cloning. The EBV-transformed cell lines from a Scott patient and the parents were described previously²⁰. The TMEM16F mRNA in these cell lines was analyzed by RT-PCR. The TMEM16F chromosomal gene was amplified by PCR from the genomic DNA of the cell lines, and directly sequenced by cycle sequencing using an ABI 3100 genetic analyzer (Applied Biosystems). The exposure of PS and PE on the cell surface was analyzed by binding of Cy5-labelled Annexin V and biotin-labelled Ro09-0198¹⁷, respectively. The internalization of NBD-PC and NBD-SM was analyzed by the BSA-extraction method essentially as described³⁰. For knock-down experiment, shRNA-retrovirus vectors for TMEM16F and control scrambled sequence were obtained from OriGene, and the resultant retrovirus was used to infect Ba/F3 cells.

Full Methods and any associated references are available in the online version of this paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at
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Author Contribution J.S. designed the experiments, carried out the experiments, and wrote the manuscript. M.U. provided biotin-labeled Ro09-0198 peptide. P.J.S. provided EBV-transformed cell lines from a patient and parents, and commented on the manuscript. S.N. was responsible for the overall study design and for writing the manuscript.

Author Information Reprints and permissions is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to S.N. (e-mail: snagata@mfour.med.kyoto-u.ac.jp).

Figure 1. Molecular cloning of TMEM16F. **a**, Ba/F3, treated with A23187 \pm CaCl₂, stained with Annexin V and PI. **b**, Ba/F3, incubated with BAPTA-AM, treated with A23187. Annexin V-profile in PI-negative population. Open, resting cells. **c**, Ba/F3, treated with A23187, then with BAPTA-AM for 5 min, stained with Annexin V. **d**, Ba/F3 and cells after 12 cycles-sorting (PS12), treated with A23187, stained with Annexin V. **e**. GFP and DsRed-profiles of PS0, PS19, and PS0/19 hybrid. Bottom, these cells, treated with A23187, stained with Annexin V. **f**, Ba/F3 transformed with PS19 cDNA library, treated with A23187, stained with Annexin V, and sorted (LD-PS0). Annexin V- and PI-profile after first (LD-PS1), and third (LD-PS3) sorting. Right, Annexin V-profile of original (LD-PS0) and after fourth sorting (LD-PS4) without A23187.

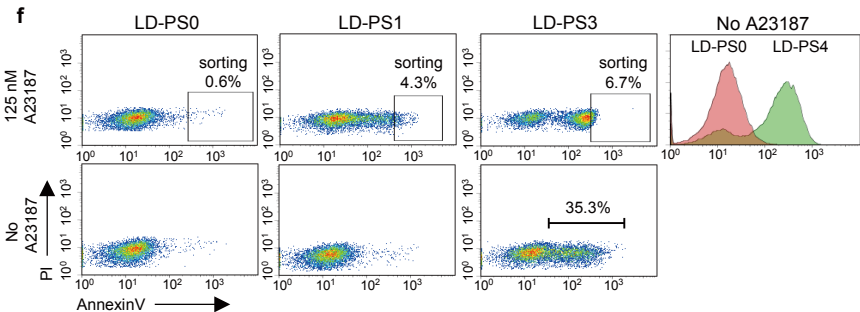
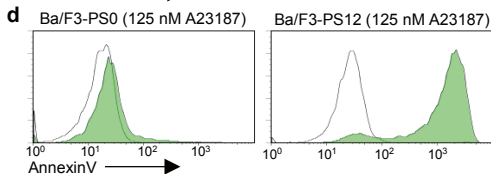
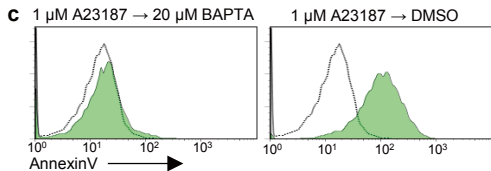
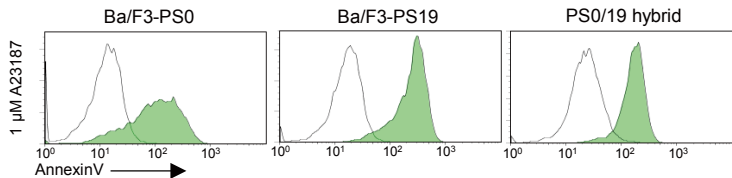
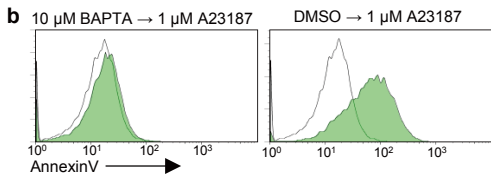
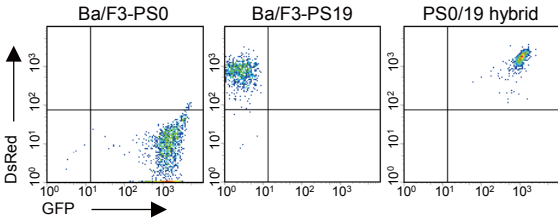
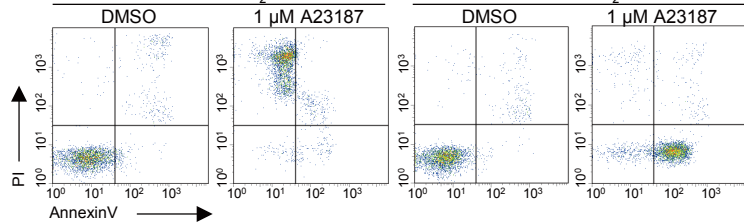
Figure 2. Phospholipids-scrambling in TMEM16F-expressing cells. a,

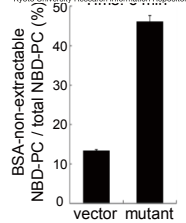
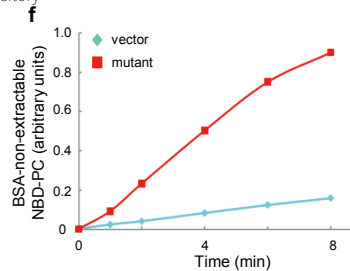
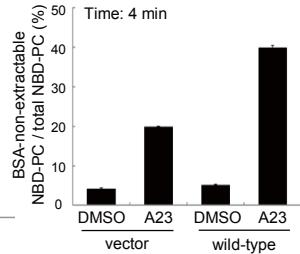
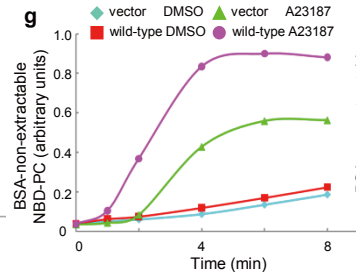
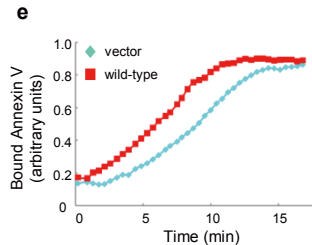
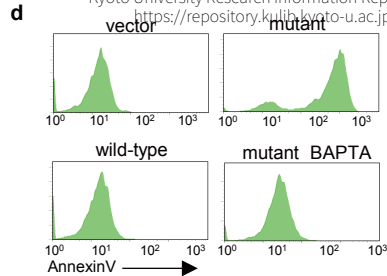
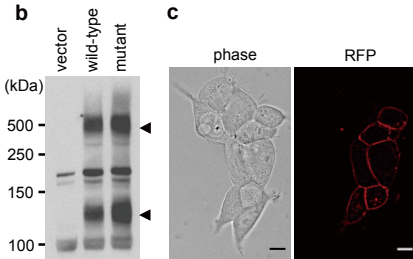
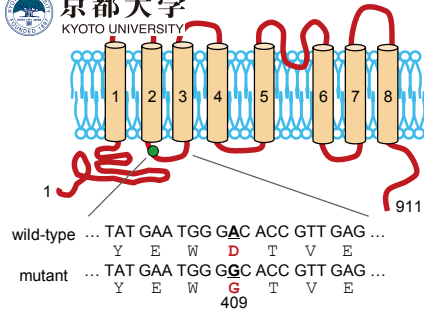
Schematic representation of mouse TMEM16F and D409G mutant. **b**, Western blotting of Ba/F3 expressing Flag-wild-type and mutant TMEM16F with anti-Flag. Arrowheads, monomer and multimer of TMEM16F. **c**, 293T expressing TMEM16F-mRFP, observed by fluorescent microscope. Scale bar, 10 μ m. **d**, Ba/F3, transformed with vector, or wild-type or mutant TMEM16F, stained with Annexin V \pm BAPTA-AM pre-treatment. **e**, Vector- or wild-type TMEM16F-Ba/F3, preincubated with Annexin V, mixed with A23187, and fluorescence monitored. y-axis, fluorescence intensity on FACS in arbitrary units. **f**, Vector- or mutant TMEM16F-expressing Ba/F3, incubated at room temperature with 0.5 μ M NBD-PC in HBSS-Ca²⁺. After dilution with fatty-acid-free BSA buffer, the fluorescence intensity determined by FACS, and shown in arbitrary units. **g**, Vector- or wild-type TMEM16F-expressing Ba/F3, preincubated at 4°C with 0.1 μ M NBD-PC. A23187 added, incubated at room temperature, internalized NBD-PC determined as above. In **f** and **g**, percentage of BSA-non extractable NBD-PC determined in triplicates, plotted with S.D. All experiments carried out at least three times.

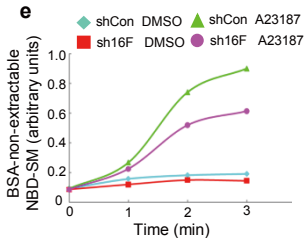
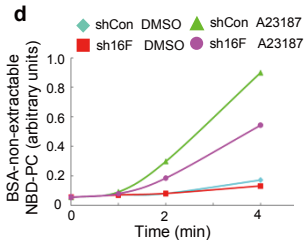
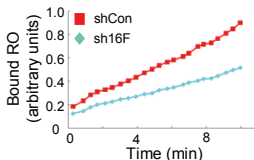
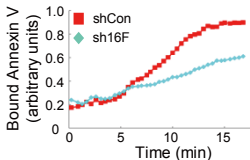
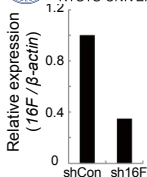
Figure 3. Requirement of TMEM16F for phospholipids-scrambling. a.

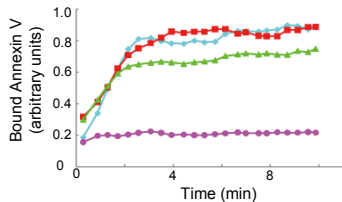
Ba/F3 transformants expressing shRNA for TMEM16F (sh16F) or scrambled shRNA (shCon). TMEM16F mRNA level, normalized to β -actin mRNA, and shown as relative expression. **b** and **c**, sh16F- or shCon-expressing Ba/F3, preincubated with Cy5-Annexin V (**b**) or biotin-Ro09-0198 and APC-streptavidin (**c**). A23187 added, and fluorescence monitored. **d** and **e**, sh16F- or shCon-expressing Ba/F3, preincubated with 0.5 μ M NBD-PC (**d**) or NBD-SM (**e**) in HBSS-Ca²⁺. A23187 added, incubated, diluted with fatty-acid-free BSA buffer, and fluorescence determined. Experiments in **b-e** carried out at least three times.

Figure 4. A splice mutation of TMEM16F gene in a Scott patient. **a**, Cells from control, Scott patient, and patient's parents, preincubated with Annexin V. A23187 added, and fluorescence monitored. **b**, RT-PCR for TMEM16F mRNA for exons 1-12 and 11-20 with RNA from the patient and parents. **c**, The junction between exon 13 and intron 12 of TMEM16F gene sequenced from 3'. The "CT" complementary to splice acceptor site "AG" underlined. Arrowheads, the mutation. **d**, RT-PCR for exons 12-16. Arrowheads, fragments for wild-type and exon 13-deleted form. **e** and **f**, Splicing in the patient's TMEM16F gene. The mutation causes skipping of exon 13, resulting in a frame-shift mutation with premature termination in 3rd transmembrane region.

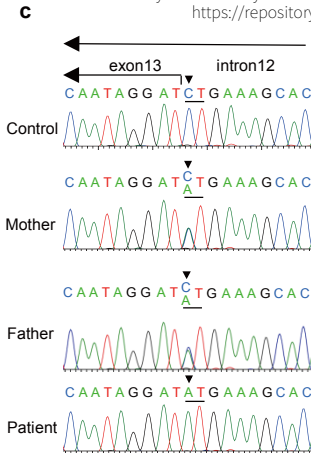
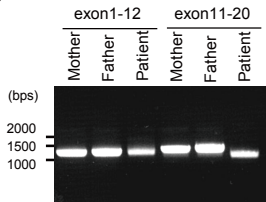




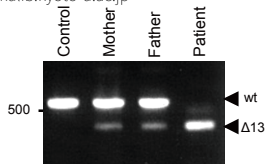




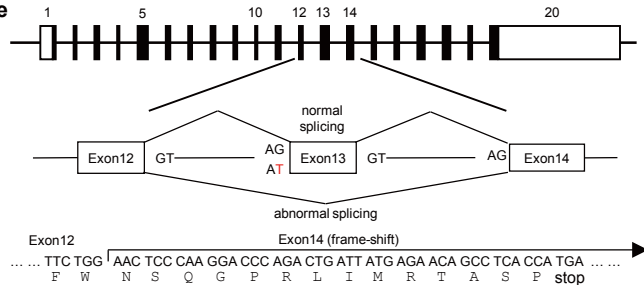
b



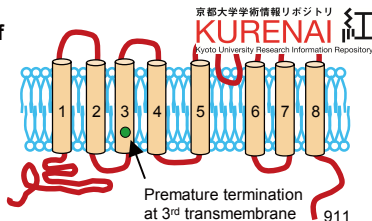
d



e



f



Methods

Cell lines, recombinant proteins, antibodies, serum, and reagents. Mouse interleukin (IL-3)-dependent Ba/F3 cells were maintained in RPMI containing 10% fetal calf serum (FCS, Gibco), 45 units/ml of recombinant mouse IL-3, and 50 μ M β -mercaptoethanol. The EBV-transformed human cell lines²⁰ from a Scott syndrome patient and her parents were grown in RPMI1640 containing 10% FCS, and 50 μ M β -mercaptoethanol. Human 293T cells and Plat-E packaging cells³¹ were cultured in DMEM containing 10% FCS. Recombinant mouse IL-3 was produced by mouse C127I cells transformed with a bovine papillomavirus expression vector bearing mouse IL-3 cDNA as described³². Biotin-labeled Ro09-0198 was prepared as described previously³³. Flag-tagged mouse MFG-E8 was produced in human 293T cells as described³⁴, and the secreted MFG-E8 was purified using anti-Flag M2 beads (Sigma-Aldrich).

$\text{Ca}^{2+}/\text{Mg}^{2+}$ -free RPMI1640 medium was purchased from Cell Science & Technology Institute. Ca^{2+} -free RPMI medium contained 0.5 mM MgSO_4 . Ca^{2+} -free FCS was prepared by dialyzing FCS for 2 days against PBS with four changes of buffer. Dynasore was purchased from Calbiochem.

BAPTA-AM (*O,O'*-Bis(2-aminophenyl)ethyleneglycol- *N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester) was from Dojindo. 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC), and N-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sphingosine-1-phosphocholine (NBD-SM) were purchased from Avanti Polar Lipids.

Treatment with Ca^{2+} -ionophore, flow cytometry, and cell sorting. To expose phosphatidylserine (PS) on the cell surface, cells (2×10^5 cells) in a 96-well microtiter plate were washed with PBS, re-suspended in 200 μ l of Hank's Balanced Salt Solution (HBSS, Gibco) and treated with A23187 (Sigma Aldrich) at 37°C for 15 min. The cells

were stained on ice for 15 min with 2500-5000-fold diluted Cy5-labeled Annexin V (Biovision) in staining buffer (10 mM HEPES-NaOH buffer [pH7.4] containing 140 mM NaCl and 2.5 mM CaCl_2) in the presence of 5 $\mu\text{g/ml}$ propidium iodide (PI). Flow cytometry was performed on a FACS Aria (BD Bioscience) or FACSCalibur (BD Bioscience), and the data were analyzed using FlowJo Software (True Star).

A subline of Ba/F3 cells that was sensitive to Ca^{2+} -ionophore-elicited PS exposure was selected by repetitive FACS sorting. In brief, after 2×10^7 Ba/F3 cells in HBSS were treated at 37°C for 15 min with A23187, they were suspended in 1 ml of Annexin V- staining buffer that had been pre-chilled at 4°C . The cells were stained with Cy5-Annexin V on ice as described above, and sorted using a FACS Aria whose injection chamber was kept at 4°C . Cells providing the highest level of Cy5-fluorescence signal (top 0.5-5.0%) were collected, and re-suspended at a density of more than 1.0×10^5 cells per ml in Ca^{2+} -free RPMI containing 5% dialyzed FCS, 45 units/ml IL-3, and 50 μM β -mercaptoethanol. Twenty-four hours later, the cells were re-suspended in normal Ca^{2+} -containing RPMI medium, and expanded for the next sorting.

Construction of the cDNA library. Total RNA was prepared from Ba/F3 PS19 cells using an RNeasy Mini Kit (Qiagen), and poly(A) RNA was purified using an mRNA Purification Kit (GE Healthcare) with two cycles of oligo(dT)-cellulose column chromatography. Double-stranded cDNA was synthesized with random hexamers as primers, using a cDNA synthesis kit (SuperScriptTM Choice System for cDNA Synthesis, Invitrogen). A *Bst* XI adaptor was attached, and the fragments were size-fractionated by electrophoresis through a 1% agarose gel (Seakem[®] GTG agarose, Lonza). DNA fragments longer than 2.5 kb were recovered from the gel using a DNA extraction kit (Wizard[®] SV Gel and PCR Clean-up System, Promega), and ligated into a *Bst* XI-digested pMXs vector³⁵. *E. coli* DH10B cells (ElectroMax DH10B; Invitrogen) were transformed by electroporation using a Gene Pulser (Bio-Rad). About 9.3×10^5

clones were produced, and plasmid DNA was prepared using a QIAfilter Plasmid Maxi Kit (Qiagen).

Cell fusion. Ba/F3-PS0 and Ba/F3-PS19 cells were transduced with pMXs-puro EGFP and pMXs-neo DsRed, respectively, and cultured in the presence of 1 μ g/ml puromycin or 1 mg/ml G418. Ba/F3-PS0 EGFP cells and Ba/F3-PS19 DsRed cells were fused in the presence of PEG1500, and cultured in the presence of 1 μ g/ml puromycin and 1 mg/ml G418. The EGFP/DsRed double-positive cells were sorted using a FACS Aria.

Screening of cDNA library. Plasmid DNA (108 μ g) from the cDNA library was introduced by lipofection using FuGENE6 (Roche Diagnostics) into 7.2×10^7 PLAT-E packaging cells³¹ grown in eighteen 10-cm dishes. Two days after the transfection, the viruses in the culture supernatant were spun down at 4°C by centrifugation at 6,000 x g for 16 h, re-suspended in RPMI1640 medium containing 10% FCS and 45 units/ml IL-3, and used to infect 7.2×10^6 Ba/F3 cells in the presence of 8 μ g/ml polybrene (Sigma-Aldrich). After a 24-h culture, the medium was replaced with fresh medium, and the cells were further cultured for 2 days. The sorting of cells that were sensitive to ionophore-induced PS exposure was performed as described above.

Isolation of cDNA fragments from Annexin V-positive Ba/F3 cells. To isolate the cDNA integrated into the retroviral vector, the genomic DNA was extracted from Ba/F3 cell transformants using the Wizard[®] Genomic DNA Purification System (Promega), and subjected to PCR using the Expand Long Template PCR System (Roche Diagnostics). The PCR primers (5'-CCCGGGGGTGGACCATCCTCT-3' and 5'-CCCCTTTTCTGGAGACTAAAT-3') carried sequences from the pMXs vector, and the conditions for PCR were 10 sec at 96°C, 30 sec at 58°C, and 4 min at 68°C for 35 cycles. The PCR fragments were cloned into the pGEM-T Easy vector (Promega), and subjected to DNA sequencing analysis using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Expression vector for TMEM16F and its mutants. The Flag-tag sequence was integrated into the *Eco* RI and *Xho* I sites of the retroviral vector pMXs-puro, resulting in pMXs-puro c-Flag. The full-length coding sequences for mouse (m)TMEM16F (GenBank NM_175344) was prepared by RT-PCR with the mRNA from Ba/F3 cells. The primers used were as follows. (in each primer, the *Eco* RI recognition sequence is underlined):

mTMEM16F, 5'-ATATGAATTCGACATGCAGATGATGACTAGGAA-3' and 5'-ATATGAATTCGACATGCAGATGATGACTAGGAA-3';

The PCR fragments were inserted into the *Eco* RI site of pMXs-puro c-Flag, and the authenticity of the cDNAs was verified by DNA sequencing.

For the expression plasmid of TMEM16F-mRFP, the coding sequence for mRFP in pcDNA-mRFP (Invitrogen) was joined in frame to the C-terminus of mouse TMEM16F, and introduced into pMXs vector.

Expression in mouse Ba/F3 and human 293T cells. The expression vector for Flag-tagged TMEM16F in pMXs-puro was introduced into PlatE cells. The produced retrovirus was concentrated as described above, and used to infect Ba/F3 to establish stable transformants. The transformants were selected by culturing the cells in medium containing puromycin (1.0 µg/ml). To express TMEM16F-mRFP, human 293T cells were transfected by the lipofection using FuGENE6 with the pMXs vector carrying the TMEM16F-mRFP sequence. One day later, the transfected cells were observed by fluorescence microscopy (BioRevo BZ-9000, Keyence).

Western blotting. Cells were lysed in RIPA buffer (50 mM Hepes-NaOH buffer [pH 8.0] containing 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, and 10% protease inhibitor cocktail [Complete Mini, Roche Diagnostics]). The lysate was mixed with 5 x SDS sample buffer [200 mM Tris-HCl (pH 6.8), 10% SDS, 25%

glycerol, 0.5% β -mercaptoethanol, and 0.05% bromophenolblue], boiled for 5 min, and separated by electrophoresis on a 10% polyacrylamide gel (Bio Craft). After the proteins were transferred to a PVDF membrane (Millipore), the membranes were probed with HRP-conjugated mouse anti-FLAG M2 (Sigma), and the peroxidase activity was detected by a Western Lightning[®]-ECL system (PerkinElmer).

RT-PCR of TMEM16F cDNA and sequencing of its chromosomal gene in Scott syndrome patient. Total RNA was prepared from EBV-transformed cell lines from a Scott patient and her parents, and from a healthy control. The RNA was reverse-transcribed using Superscript III (Invitrogen), according to the manufacture's protocol, and the TMEM16F cDNA was analyzed by PCR using the following sets of primers (in each primer, the sequence containing the additional restriction site is underlined). Ex1-FW (5'-ATATGAATTTCGACATGAAAAAGATGAGCAGGAA-3') and Ex11/12-RV (5'-GCGTTCTTCTTCTGAGTAA-3');

Ex11/12-FW (5'-TTACTCAGGAAGAAGAACGC -3') and

Ex20-RV (5'-ATATGAATTCTTCTGATTTTGGCCGTAAAAT-3');

Ex12-FW (5'- TCTGTGCCAGTGCTGTCTTT-3') and

Ex16-RV (5'- CTGCAGATGGTAGTCCTGTT-3').

For the sequence analysis of the human TMEM16F chromosomal gene, genomic DNA was prepared from human cell lines, and a 964-bp DNA fragment carrying the 226-bp exon 13 and its 5'- and 3'-flanking regions (about 370-bp each) was amplified by PCR using the following primers: 5'-CCAGAGTATGCTACTAGTTG-3' and 5'-TCTCAGCAACCGAGGAACAT-3'. The PCR products were purified with a Wizard[®] SV PCR and Gel Clean-up System. Cycle sequencing was performed using a BigDye

Terminator v3.1 Cycle Sequencing kit with a primer of 5'-

ACATATGTGGATGCGCCTTC-3', and analyzed by an ABI PRIZM 3100 Genetic Analyzer.

Analysis of exposure of PS and PE. To analyze the exposure of PS and PE, cells (1.0×10^5) at the early exponential phase were washed with PBS, suspended in 1.0 ml of cold Annexin V-staining buffer with 2500-5000-fold diluted Cy5-labeled Annexin V or 800-diluted biotin-Ro09-0198³³ followed by 1.0 $\mu\text{g/ml}$ APC-labeled streptavidin, and 5 $\mu\text{g/ml}$ PI. The samples were incubated on ice for 15 min, and flow cytometry was performed on a FACS Aria or FACSCalibur as described above. For binding of MFG-E8, the cells were suspended in RPMI1640 containing 10% FCS, and incubated on ice for 20 min with 0.4 $\mu\text{g/ml}$ of the Flag-tagged D89E mutant of MFG-E8³⁴. The cells were washed with the above medium, and incubated on ice for 20 min with 1.0 $\mu\text{g/ml}$ hamster mAb against mouse MFG-E8 (clone 2422), followed by incubation with PE-labeled mouse anti-hamster IgG (BD Bioscience), and analyzed by flow cytometry with a FACS Aria.

To study the requirement of intracellular Ca^{2+} , cells (1.0×10^5) were incubated with 10 μM BAPTA-AM in RPMI1640 medium containing 10% FCS at 37°C for 5 min for the PS-exposure or for 60 min for the PE-exposure. The cells were washed with Annexin V-staining buffer, and stained with Cy5-Annexin V or biotin-Ro09-0198 as described above.

For the kinetic study of the Ca^{2+} -induced PS and PE exposure, cells (1.0×10^6) were washed with PBS, suspended in 1.0 ml of cold Annexin V-staining buffer with Cy5-labeled Annexin V or a mixture of biotin-Ro09-0198 and APC-labeled streptavidin, and 5 $\mu\text{g/ml}$ PI. Cells were mixed on ice with A23187 at a final concentration of 0.25 or 0.5 μM , and applied to the injection chamber of a FACS Aria that was set at 20°C (for Ba/F3 cells) or 37°C (for human cell lines) to induce the A23187 reaction. Data were

recorded for the indicated periods and the PI-positive cells were excluded from the analysis.

Internalization of NBD-PC, and -SM. The internalization of NBD-lipid analogies was analyzed by flow cytometry essentially as described by Williamson et al.⁸ In brief, cells (10^6 cells) were washed with HBSS, and resuspended in 0.5 ml of HBSS containing 2 mM CaCl_2 (HBSS-Ca). An equal volume of HBSS-Ca and 1 μM NBD-PC, or NBD-SM was added to the cell suspension, and incubated at room temperature. At each time point, 150 μl of cell suspension was collected, mixed with 150 μl of the prechilled (4°C) HBSS containing 5 mg/ml fatty-acid free BSA (Sigma-Aldrich) to extract the unincorporated fluorescent lipids and 500 nM Sytoxblue (Molecular Probes). To measure the total fluorescence, samples were mixed with HBSS in the absence of BSA. After incubation for 10 min at 4°C to extract the lipid, the cells were analyzed by a FACSaria for forward scatter, side scatter, log green fluorescence (NBD), and Sytoxblue fluorescence. The Sytoxblue-positive dead cells were excluded from the analysis. The data were analyzed with FlowJo Software (Tree Star). The fluorescence of NBD-phospholipids that was resistant to the BSA-extraction was regarded to be incorporated into cells.

To examine the effect of the Ca^{2+} ionophore, cells (5×10^5 cells) were washed with HBSS, resuspended in 0.5 ml of cold HBSS-Ca, and incubated on ice for 7 min. Cold HBSS (0.5 ml) containing 0.2 μM NBD-PC, or NBD-SM was added to the cell suspension and incubated further on ice for 3 min. The cells were then mixed with A23187, and incubated at room temperature to induce lipid incorporation. A 150- μl aliquot was used to determine the incorporated lipid quantity as described above.

Thin-layer chromatography. After incubation of cells with NBD-PC or NBD-SM, the phospholipids were extracted from the cells with a mixture of chloroform and methanol (2:1, v/v). The phospholipids were separated by thin-layer chromatography on a silica

gel 60 plate (Merck) using chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5 in volume) as a solvent. The fluorescence on the plate was detected by a LAS4000 image analyzer (Fuji Film).

Intracellular Ca^{2+} and Ca^{2+} influx. To determine the intracellular Ca^{2+} concentration, cells (1.0×10^6 cells) were suspended in HBSS, incubated at 37°C for 10 min with $0.4 \mu\text{M}$ Fluo-4-AM (Molecular Probes), washed with HBSS, and analyzed by FACS Aria.

The Ca^{2+} influx was measured as described³⁶. In brief, cells (1.0×10^6) were labeled with Fluo-4-AM ($1 \mu\text{M}$) in RPMI containing 10% FCS for 30 min at 37°C . After washing with the Annexin V staining buffer, the cells were kept at 4°C in Annexin V-staining buffer. The Ca^{2+} ionophore A23187 was added to the mixture at a final concentration of $0.5 \mu\text{M}$, and the change in mean fluorescence intensity (MFI) was directly recorded using FACSCalibur system. The data was analyzed with FlowJo Software.

shRNA. shRNA expression plasmids for mouse TMEM16F in a pRS shRNA vector carrying the puromycin-resistance gene were purchased from OriGene. The target sequence of the shRNA for TMEM16F was 5'-CATCTACTCTGTGAAGTTCTTCATTTTCCT-3'. The scrambled non-effective shRNA (5'-GCACTACCAGAGCTAACTCAGATAGTACT-3') in pRS was from OriGene. Ba/F3 cells were infected with retrovirus containing the shRNA, and cultured in the presence of $1.0 \mu\text{g/ml}$ puromycin. The puromycin-resistant cells were subjected to cloning by limited dilution. The TMEM16F mRNA was quantified by real-time PCR, and the clones that showed the reduced expression of TMEM16F were used for further study.

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Supplementary Figures

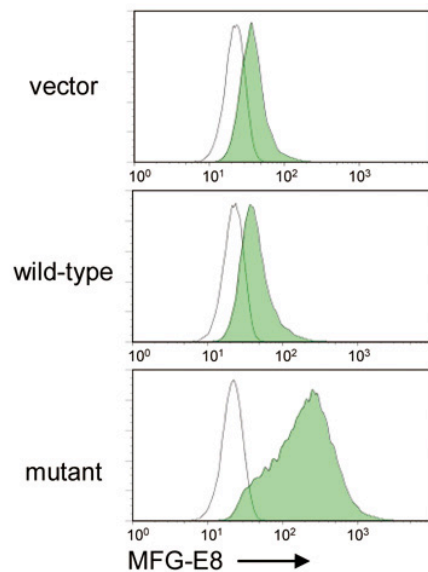


Figure 1. Constitutive binding of MFG-E8 to Ba/F3 cells expressing the D409G mutant TMEM16F. Ba/F3 cells were infected with the empty, wild-type TMEM16F- or D409G mutant TMEM16F-carrying retrovirus vector. The cells were incubated with 0.4 μ g/ml of mouse MFG-E8, followed by successive incubations with hamster anti-mouse MFG-E8 and PE-labelled mouse anti-hamster IgG as described in Methods. The cells were analyzed by flow cytometry using FACSARIA, and the staining profile is shown (filled area). The staining procedure was also performed without MFG-E8, and its profile is shown in open area.

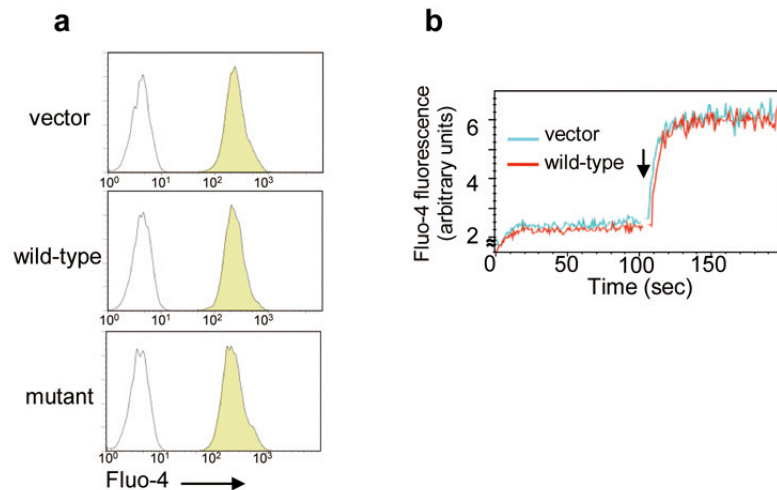


Figure 2. No effect of TMEM16F on the Ca^{2+} influx. **a**, The vector-, wild-type-, or D409G mutant TMEM16F-transformed Ba/F3 cells were loaded with Fluo-4-AM, and analyzed by FACS. Open area represents the profile without Fluo-4-AM. **b**, The vector- or wild-type TMEM16F-transformed Ba/F3 were preincubated with Fluo-4-AM. A23187 was added at the point indicated by an arrow, and the fluorescence was monitored.

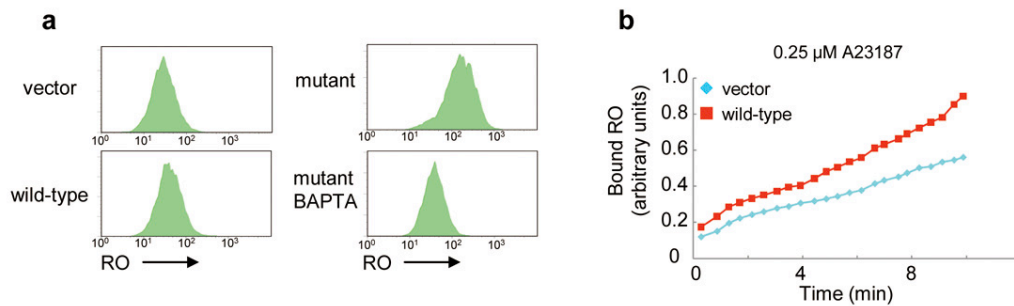


Figure 3. Effect of TMEM16F on the exposure of phosphatidylethanolamine. **a**, Ba/F3 cells transformed with vector-, wild-type-, or D409G-mutant TMEM16F were incubated with biotin-labeled Ro09-0198 with or without BAPTA-AM pre-treatment, followed by staining with APC-streptavidin. **b**, Ba/F3 cells transformed with vector or the wild-type TMEM16F were preincubated with biotin-Ro09-0198 and APC-streptavidin. A23187 was added, and the fluorescence was monitored by flow cytometry. The y-axis is fluorescence intensity observed by FACS analysis, and shown in arbitrary units.

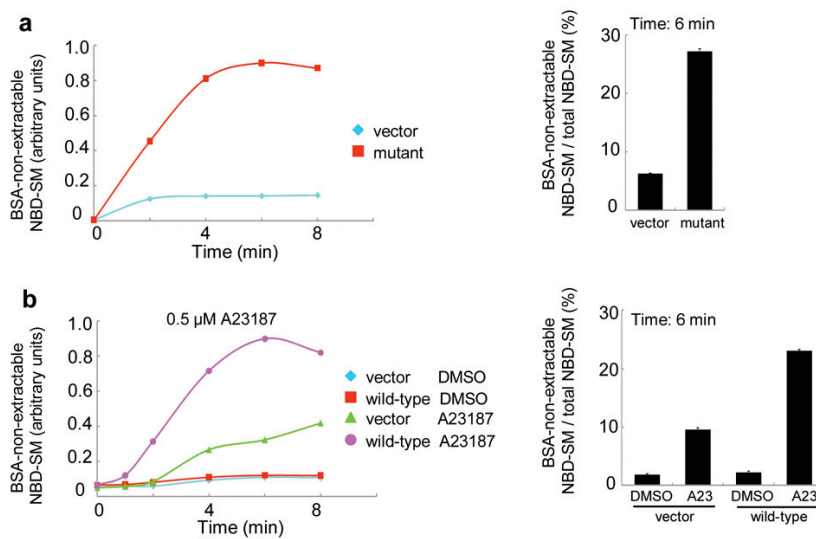


Figure 4. Effect of TMEM16F on the internalization of sphingomyelin. **a**, The Ba/F3 cells transformed with the vector or mutant TMEM16F were incubated at room temperature with 0.5 μ M NBD-SM in HBSS containing Ca^{2+} . At the indicated time, samples were diluted with cold, fatty-acid-free BSA-containing buffer, and analyzed by flow cytometry. The y-axis is fluorescence intensity observed by FACS analysis, and shown in arbitrary units. **b**, Vector- or wild-type TMEM16F-expressing Ba/F3 cells were preincubated at 4°C with 0.1 μ M NBD-SM. A23187 was added, and the NBD-SM internalized at room temperature was analyzed as above. In **a** and **b**, the percentage of NBD-SM that could not be extracted by BSA at the indicated time point was determined in triplicates, and plotted with S.D. The experiments were independently performed at least three times.

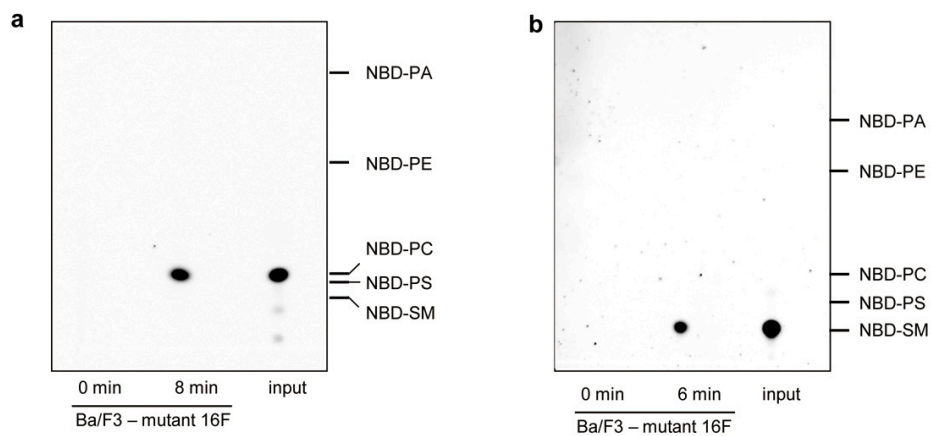


Figure 5. No breakdown of NBD-PC and NBD-SM incorporated in the cells. NBD-PC (a) or NBD-SM (b) was added to Ba/F3 expressing the D409G mutant of TMEM16F, incubated at room temperature for 6 or 8 min, and treated with fatty-acid-free BSA. The phospholipids incorporated into the cells were extracted from the cells, and analyzed by thin-layer chromatography together with the input NBD-PC and NBD-SM. As reference, NBD-derivatives of phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and sphingomyelin (SM) were chromatographed in parallel, and their positions on the chromatogram are indicated on right.

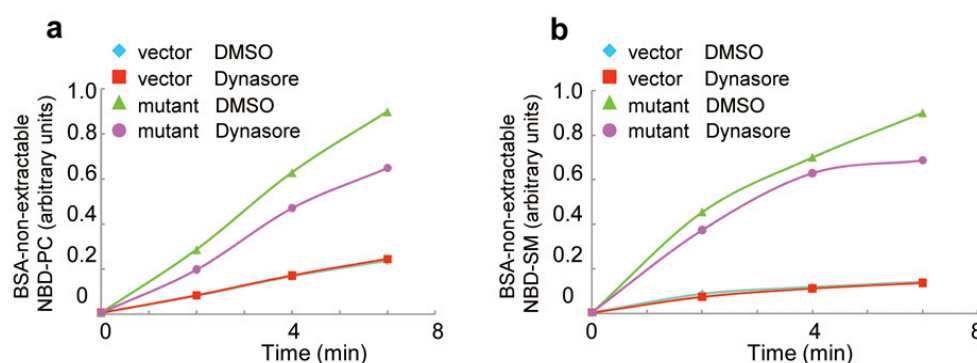


Figure 6. No inhibition of the phospholipid-internalization by an inhibitor of the endocytosis. The Ba/F3 cells transformed with the vector or D409 mutant TMEM16F were preincubated at 37°C for 30 min with or without 20 μ M dynasore in the culture medium. After washing with HBSS, the cells were incubated at room temperature with 0.5 μ M NBD-PC (**a**) or NBD-SM (**b**) in HBSS containing Ca^{2+} in the presence or absence of 20 μ M dynasore. At the indicated time, samples were diluted with cold, fatty-acid-free BSA-containing buffer, and analyzed by flow cytometry. The y-axis is fluorescence intensity observed by FACS analysis, and shown in arbitrary units.

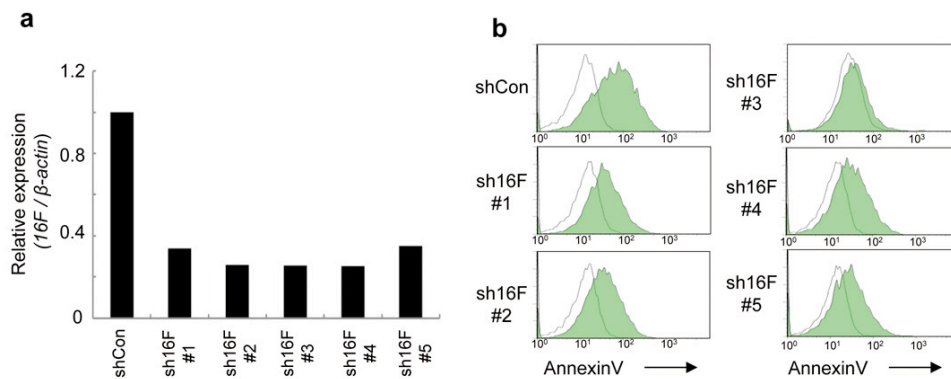


Figure 7. The effect of knock-down of TMEM16F on the Ca^{2+} -dependent PS exposure. The pRS shRNA vector for TMEM16F was introduced into Ba/F3 cells. Among the Ba/F3 stable transformant clones in which the TMEM16F mRNA level is reduced, 5 clones (sh16F #1-5) were picked and studied. As a control, a Ba/F3 cell transformant (shCon) established with pRS shRNA vector carrying a scrambled non-effective sequence was also analyzed. **a**, The TMEM16F mRNA level in the Ba/F3 transformant clones (sh16F #1-5) was determined by real-time PCR, and is shown to relative to that in the control Ba/F3 (shCon). **b**, The Ba/F3 cell transformants (shCon and sh16F #1-5) were treated with 1.0 μM A23187 at 37°C for 15 min, stained with Cy5-labelled Annexin V, and analyzed by flow cytometry. The transformants were also stained with Annexin V without the A23187-treatment, and their staining profile is shown in open area.